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Reduced Ets domain-Containing Protein Elk1 Promotes Pulmonary Fibrosis via Increased Integrin αvβ6 Expression

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Keywords: Integrin, fibrosis, Elk1, gene regulation, lung

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic lung disease with high mortality. Active Transforming Growth Factor β1 (TGFβ1) is considered central to the pathogenesis of IPF. A major mechanism of TGFβ1 activation in the lung involves the epithelially restricted αvβ6 integrin. Expression of the αvβ6 integrin is dramatically increased in IPF. How αvβ6 integrin expression is regulated in pulmonary epithlium is unknown. Here we identified a region in the β6 subunit gene (*ITGB6)* promoter acting to markedly repress basal gene transcription, which responds to both the Ets domain-containing protein Elk1 (Elk1) and the glucocorticoid receptor (GR). Both Elk1, and GR, can regulate αvβ6 integrin expression *in vitro*. We demonstrate Elk1 binding to the *ITGB6* promoter basally, and that manipulation of Elk1, or Elk1 binding, alters *ITGB6* promoter activity, gene transcription and αvβ6 integrin expression. Crucially, we find that loss of Elk1 causes enhanced *Itgb6* expression and exaggerated lung fibrosis in an *in vivo* model of fibrosis, whereas the GR agonist Dexamethasone inhibits *Itgb6* expression. Moreover Elk1 dysregulation is present in epithelium from patients with IPF. These data reveal a novel role for Elk1 regulating *ITGB6* expression and highlight how dysregulation of Elk1 can contribute to human disease.

INTRODUCTION

The prognosis of idiopathic pulmonary fibrosis (IPF) patients is poor, with 5-year survival rates worse than most cancers [1] and the incidence is rapidly increasing [2]. IPF is characterised by excessive matrix deposition within the lung interstitium leading to deteriorating pulmonary function and ultimately death. IPF pathogenesis is poorly understood, however, the current paradigm suggests that recurrent epithelial injury and failing repair promotes expansion and transdifferentiation of myofibroblasts within the interstitium [3]. Improved understanding of the mechanisms driving fibrogenesis is critical for the development of urgently needed new therapeutics.

Transforming growth factor β1 (TGFβ1) is a key cytokine implicated in the development of fibrosis [4]. It has profound effects on both epithelial cells and fibroblasts, two of the key effector cells in fibrogenesis, and mediates many of the characteristic pathologic features of fibrosis including epithelial cell apoptosis,

myofibroblast transdifferentiation and subsequent collagen production [5, 6]. For TGFβ1 to exert any biological effect it must first be activated, and activation by the αvβ6 integrin is an important feature in experimental models of fibrosis in multiple organ systems [7-12].

Fundamentally, high levels of αvβ6 integrins have been demonstrated in lung tissue from patients with pulmonary fibrosis [9, 13] and are associated with a worse prognosis [14]. Similarly, levels of β6 subunit mRNA (*ITGB6*) correlate with increasing severity of fibrosis in the liver [15]. The molecular mechanisms regulating expression levels of αvβ6 integrins are, therefore, likely to be critical for ensuring normal repair in response to injury rather than pathological fibrosis. Such mechanisms, however, have not been investigated in detail.

It has previously been shown that TGFβ1 increases *ITGB6* levels and elevates expression of αvβ6 integrins by guinea pig airway epithelial cells *in vitro* [16] and a positive feedback loop of αvβ6-mediated TGFβ1 activation promoting enhanced integrin expression in the lung has been proposed [17]. It is possible that TGFβ1-independent pathways may also contribute to *ITGB6* regulation. For example a potential role for ets-1 during increased transcription of the *ITGB6* gene has been suggested [18] and over-expression of active Stat3 leads to increased *ITGB6* expression in carcinogenesis [19]. However, whether these mechanisms are involved in upregulation of *ITGB6* in pulmonary fibrosis is unknown.

This study aimed to investigate the molecular mechanisms driving *ITGB6* expression in lung epithelial cells, and investigate the importance of these pathways in fibrogenesis. We have identified a novel region of the *ITGB6* promoter responsible for repressing transcription and demonstrate that Elk1, as well as the glucocorticoid receptor (GR), can act to repress the *ITGB6* gene. We demonstrate that Elk1 deficiency *in vivo* enhances *Itgb6* expression and exacerbates bleomycin-induced fibrosis in mice, whereas the GR agonist Dexamethasone represses *Itgb6* expression. Finally, we show that lung tissue from pulmonary fibrosis patients displays reduced Elk1 expression, associated with reduced Elk1 binding to the *ITGB6* promoter. These data highlight a novel and crucial function of the transcription factor Elk1 in modulating αvβ6 expression in the lung.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents

Immortalised human bronchial epithelial cells (iHBECs; a kind gift from Professor Jerry Shay, University of Texas) or small airway epithelial cells (SAECs; Lonza, UK) were used for many *in vitro* experiments. The iHBEC immortalised cell line was selected over primary alveolar epithelial cells for the majority of *in vitro* studies due to the technical advantages in performing molecular mechanistic studies such as transfections and ChIP in these cells. iHBECs retain their ability to differentiate into basal, mucin-producing and ciliated epithelial cells [20]. Furthermore, iHBECs are one of the only immortalised or transformed lung epithelial cell lines that retains their αvβ6 integrin expression *in vitro.* iHBECs were cultured in keratinocyte serum free medium (K-SFM; Lonza, UK) containing bovine pituitary extract (Lonza, UK), epidermal growth factor (Lonza, UK), G418 (Sigma-Aldrich, UK), puromycin (Sigma-Aldrich, UK), penicillin and streptomycin (both from Sigma-Aldrich, UK).

Primary small airway epithelial cells (SAECs) were used to confirm some findings obtained in iHBECs. They were cultured in small airway growth media (Lonza, UK) containing the supplied supplements. All cells were growth arrested in supplement-free media for 24 hours prior to the start of experiments, except in the case of transfection experiments. To assess binding of endogenous Elk1 to exogenous *ITGB6* promoter constructs we used the adenosquamous carcinoma cell line H647 after determining that these cells exhibit high expression level of endogenous Elk1. H647 cells were cultured in Roswell Park Memorial Institute (RPMI; Sigma Aldrich, UK) media with 10% foetal calf serum (FCS), L-glutamine, penicillin and streptomycin.

Elk1 and GAPDH antibodies for western blotting were supplied by Cell Signalling Technology, UK. All siRNA constructs and the associated reagents were provided by Santa Cruz Biotechnology, USA. Formaldehyde, glycine, dexamethasone and progesterone were supplied by Sigma Aldrich, UK. PE-labelled or FITC-labelled secondary antibodies for flow

cytometry, TriZol reagent, zysorbin, TA cloning kit and T4 ligase were supplied by Invitrogen, UK. All reagents required for the synthesis of cDNA from RNA, including Moloney murine virus reverse transcriptase, and all plasmids not provided by Addgene or a collaborator plus Transfast reagent were provided by Promega, UK. Kapa Taq polymerase for use in QPCR reactions was supplied by Kapa Biosystems, USA. All ChIP antibodies were obtained from Abcam, UK.

Flow Cytometry

Flow cytometry was used to assess αvβ6 cell surface expression as previously described [13]. Briefly, iHBECs were labelled with 10μg anti-αvβ6 antibody (clone 6.3G9) [21] and a PE-labelled anti-mouse secondary antibody. A secondary antibody only negative control was performed. Fluorescence was assessed using a BD FACSDIVA flow cytometer.

Quantitative Polymerase Chain Reaction (Q-PCR)

Gene expression of both human and murine genes was measured using an MxPro3000 instrument (Stratagene, CA, USA) and Kapa Taq using the following primer sequences: human *ITGB6* sense 5'-AAACGGGAACCAATCCTCTGT-3' and anti-sense 5'- GCTTCTCCCTGTGCTTGTAGGT-3', human β2-microglobulin (*B2M)* 5'-AATCCAAATGCGGCATCT-3' and anti-sense 5'-GAGTATGCCTGCCGTGTG-3', human *ELK1* sense 5'-CCACCTTCACCATCCAGTCT-3' and anti-sense 5'-TCTTCCGATTTCAGGTTTGG-3', murine *Itgb6* sense 5'-TCTGAGGATGGAGTGCTGTG-3' and anti-sense 5'-GGCACCAATGGCTTTACACT-3'. . All QPCR reactions were performed at an annealing temperature of 60°C. Amplification of a single DNA product was confirmed by melting curve analysis. Data were expressed as relative expression using the ΔΔCt equation.

Generation of *ITGB6* Promoter Deletion Mutant Series

The 1.1kb insert was excised from pGL2-*ITGB6* [18] and ligated into pGL3 vector (Promega, UK) using T4 ligase then sequenced to verify presence of insert. Progressively truncated forms of the reporter (see Figure 1A) were amplified by PCR and TA cloned with a commercially available kit (Invitrogen, UK). The purified inserts were then ligated into the pGL3 vector using T4 ligase (Invitrogen, UK) and sequenced to confirm the presence of the correct insert.

Reporter Construct Transfections

Transient transfections were performed using Transfast (Promega, UK) transfection reagent using 0.75μg reporter plasmid DNA with 7.5ng renilla luciferase DNA at a 1:2 DNA: Transfast ratio. Briefly, cells were seeded at $2x10^5$ cells/ml then cultured for 8 hours in supplemented KSFM media prior to transfection overnight in unsupplemented KSFM media. The following day cells were stimulated as required for the experiment. To assess of binding of endogenous Elk1 to *ITGB6* reporter constructs 0.75μg reporter plasmid DNA was transfected in to H647 cells at a 1:2 DNA: Transfast ratio for 24 hours.

siRNA Transfections

siRNA directed towards *Elk1, GR* or a control siRNA were transfected into iHBECs using the siRNA transfection reagents and protocol provided by Santa Cruz Biotechnology. 40pmol of siRNA was used per well of cells in a 24 well plate. Cells were transfected for 24 hours prior to the start of an experiment to allow sufficient knockdown of target protein.

Site Directed Mutagenesis (SDM)

Site directed mutagenesis was performed using the QuickChange II Site Directed Mutagenesis kit (Agilent, UK) according to the manufacturer's instructions. The Elk1 binding sites located at -772 (reverse strand) and -752 were mutated from GGAA to AAGA using the following primers: sense 5'-CCCCCAGGGTGTTCTTCAAAGAGGTGTTTTGAGATTTGTAGAGTC-3' and anti-sense 5'-GACTCTACAAATCTCAAAACACCTCTTTGAAGAACACCCTGGGGG-3'. All constructs were sequenced prior to use in experiments to confirm the presence of the correct mutation.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) allowed the binding of proteins to the *ITGB6* promoter to be determined. The ChIP-IT Express kit (Active Motif, UK) was used to assess binding in

cultured cells as previously described for airway smooth muscle cells [22]. Briefly, cells were fixed with 1% formaldehyde for 5 minutes and lysed prior to shearing of the cellular chromatin by sonication using an Epishear sonicator (Active Motif, UK). 25µg total chromatin was immunoprecipitated with 10µg antibody and Protein G magnetic beads. ChIP QPCR data were normalised to input chromatin (i.e. the amount of total chromatin present in the IP sample) then expressed as relative binding compared with control sample using the ΔΔCt equation. ChIP QPCR primer sequences were as follows using an annealing temperature of 60°C: *ITGB6* promoter -934 to -753 sense 5'-CATGCTTACCCAGGAATGCT-3' and anti-sense 5'-ACACCCTGGGGGAAAAATAC-3', *ITGB6* promoter -1604 to -1497 sense 5'-ACTGCCCTCCAGCCTAGAA-3' and anti-sense 5'-TTGACAGAAACTAAATGCCCAA-3'.

ChIP on human lung tissue was performed by first creating a single cell suspension by passing the tissue through a 100µm cell strainer. The cellular chromatin was then cross-linked in 1% formaldehyde for 5 minutes. The crosslinking was reversed with glycine, the cells lysed in 10% SDS, and then the chromatin was sheared by sonication using an Epishear sonicator (Active Motif, UK). 100µg of total tissue chromatin was subjected to immunoprecipitation with 10µg of Elk1 antibody overnight. Antibody-bound DNA was extracted using Zysorbin.

ChIP was employed to assess binding of endogenous Elk1 protein to exogenous *ITGB6* promoter plasmid constructs. H647 cells were used, which we found to express high levels of endogenous Elk1 (data not shown). Following transfection and expression of exogenous constructs the cells were formaldehyde fixed and processed as stated above for other cultured cells. QPCR of the resulting immunoprecipitated (IP) DNA was performed using primers directed against the luciferase sequence of the pGL3 plasmid in order to assess Elk1 binding only to exogenous DNA, not endogenous DNA (sense 5'-GCTTTTACAGATGCACATATC-3' and anti-sense 5'-CCATACTGTTGAGCAATTCACG-3'). Relative binding was normalised to input chromatin luciferase critical threshold (ct) signal (i.e. amount of luciferase plasmid DNA present) to ensure differences in transfection efficiency between the constructs did not affect detection of Elk1 binding levels. Data were expressed as relative binding to the non-mutated *ITGB6* promoter construct using the ΔΔCt equation.

Western Blots

Human lung tissue was homogenised using a mechanical tissue tearer in ice-cold lysis buffer (20mM Tris-HCl, 137mM NaCl, 1% Triton X-100, 2mM EDTA, 10% glycerol – all supplied by Sigma-Aldrich, UK) supplemented with protease and phosphatase inhibitors (Complete Mini protease inhibitor tablet and Phos-stop inhibitor tablets; Roche, UK). Expression of Elk1, GR and GAPDH proteins was measured by western blotting as previously described for Smad2/3 [23]. Briefly, protein concentrations in each sample were determined using a bicinchroninic assay (BCA; Pierce, UK) according to the manufacturer's instructions and using increasing concentrations of bovine serum albumin as a standard curve. 100µg of total protein was loaded in to each lane of an SDS-PAGE gel and a current of 150V was applied for 60 minutes. The gel was transferred on to a polyvinylidene membrane, blocked with 5% non-fat milk, then probed with the following antibodies overnight: rabbit anti-Elk1 (Cell Signalling Tehcnology, UK, #9182) at 1:1000 dilution, rabbit anti-GR (Cell Signalling Technology, UK, #3660) at 1:1000 dilution, goat anti-GAPDH (V-18; Santa Cruz Biotechnology, USA) at 100ng/ml. All horseradish peroxidase conjugated secondary antibodies were used at a 333µg/ml and were purchased from Dako, UK. Western blot films were scanned and band density calculated by inverting the image and measuring mean grey pixels using Adobe Photoshop CS5 Extended (32 bit, Version 12.1).

***In vivo* Bleomycin Model of Pulmonary Fibrosis**

Male *Elk1*$^{-/0}$ [24] and wild-type mice (aged 5-7 weeks) were treated with either 0 or 60 IU bleomycin sulphate in 50 µl saline via the oropharyngeal route under isofluorane anaesthesia (2.5%, 2 L/min flow of oxygen). All tissue was collected 28 days post bleomycin treatment. The lungs were either excised and snap-frozen at -80°C for mRNA and hydroxyproline assessment, or insufflated with formalin at constant gravitational pressure (20

cm H_2O) then paraffin wax embedded for histology and immunohistochemistry.

To assess the role of GR in regulating *Itgb6* mRNA *in vivo* male 6 week old C57/black6/J mice were treated with either 60IU bleomycin in 50µl saline, or saline only as a control, via the oropharyngeal route under isoflourane anaesthesia (2.5%, 2 L/min). All animals also received daily treatment with either 1mg/kg dexamethasone (100µl volume) or PBS only as a control via intraperitoneal injection. After 14 days the lungs were removed and snap-frozen at -80°C for subsequent RNA analysis.

Immunohistochemistry

5 µm thick sections of paraffin wax embedded lung tissue of murine and human origin was subjected to immunohistochemistry to evaluate expression levels of the αvβ6 integrin as previously described [13]. An antibody directed against murine αvβ6 (clone ch2.A1) and an antibody directed against human αvβ6 (clone 6.3G9) were kindly provided by Biogen Idec, USA. Murine tissue from the bleomycin model of pulmonary fibrosis was also subjected to immunohistochemistry to detect levels of pSmad2. Tissue sections were boiled in 10 mM citrate buffer to retrieve endogenous antigens then exposed to anti-pSmad2 antibody (1:500 dilution, Cell Signalling, UK) overnight. Staining was visualised with DAB as for αvβ6.

Human lung tissue from four fibrotic and four non-fibrotic donors was subjected to immunohistochemistry to detect levels of either αvβ6 (see above for αvβ6) or Elk1. For Elk1 tissue was dewaxed and rehydrated and then boiled in 10 mM citrate buffer. Following blocking in 5% donkey serum the sections were incubated overnight with either Elk1 antibody (1.1µg/ml, Abcam clone E277). Staining was visualised using DAB. All staining was visualised using a Nikon 90i light microscope and NIS-Elements image acquisition software.

Quantification of pSmad2 Positive Nuclei

Quantification was performed using a Nikon Eclipse 90i microscope and Nikon NIS Elements software. Five random fields of view at x20 magnification were observed per tissue section. All brown nuclei present in the the field of view were counted.

Masson's Trichrome Histological Staining

5 µm thick histological sections of lung tissue were dewaxed and rehydrated using xylene and decreasing concentrations of ethanol. The tissues were incubated overnight at room temperature in Bouin's solution. The lung tissue was then subjected to consecutive histological staining in Weigert's solution (5 minutes), Biebrich scarlett acid fuchsin (5 minutes), phosphomolybolic acid/ phototungstic acid (seven minutes), analine blue (5 minutes) and 1% acetic acid (1 minute). The sections were then dehydrated and imaged using Nikon Eclipse 90i microscope.

Isolation of mRNA from Lung Tissue

Human and murine lung tissue was ground to powder in liquid nitrogen using a pestle and mortar. Frozen lung tissue was then added to TriZol reagent (1 ml TriZol per 0.5 g tissue) and incubated on ice for 5 minutes prior to the addition of chloroform (200 µl/ml Trizol). The samples were centrifuged at 11,000 g for 20 minutes to allow phase separation then the aqueous layer transferred to a new tube. RNA was precipitated by addition of 500 µl isopropyl alcohol and 1 µl glycogen and centrifuged. The RNA was washed in 75% ethanol prior to being re-suspended in nuclease free water.

Hydroxyproline Assay

Hydroxyproline levels in murine lung tissue were measured as previously described [25]. Briefly, lung tissue was hydrolysed in 6 N HCl at 100°C overnight and the hydrosylate resuspended in water. 1.4% chloramine T in 10% isopropanol and 0.5 M sodium acetate were added for 20 minutes, followed by Erlich's solution for 15 minutes at 65°C. Absorbance was measured at 550 nm.

Human Tissue and Ethical Approval

Lung tissue from pulmonary fibrosis patients (PF) was obtained either post mortem or from lung transplant patients following informed, written consent and ethical review (Ethical approval numbers: Nottingham Respiratory Research Unit 08/H0407/1; Papworth Hospital Research Tissue Bank, REC: 08/H0304/56; UCSF IRB #10-00198, South East Scotland SAHSC Bioresource 06/S1101/41). In all cases the pathological diagnosis was usual interstitial pneumonia and the clinical diagnosis IPF was

made based on ATS/ERS consensus criteria [26]. Non-fibrotic human lung tissue was obtained from non-cancerous tissue removed during surgery or from donor lungs unsuitable for transplant. All experiments were performed in accordance with the WMA Declaration of Helsinki.

Animal studies using bleomycin were approved by the University of Nottingham Ethical Review Committee and were performed under Home Office Project and Personal License authority within the Animals (Scientific) Procedures Act 1986. Animals received free access to food and water at all times.

Statistics

All cell-based experiments were repeated three times and expressed as mean data from the three independent experiments. Statistical significance was determined by either T test when comparing two data sets or ANOVA for comparing multiple data sets. Data from experiments comparing pulmonary fibrosis (PF) and control non-fibrotic (NF) human tissues were not normally distributed therefore the non-parametric Mann Whitney U test was used to determine significance. $P<0.05$ was accepted as significant in all cases.

RESULTS

The *ITGB6* gene promoter contains a putative repressor region

To investigate the presence of transcriptional regulatory regions within the *ITGB6* gene promoter we created a series of truncated *ITGB6* promoter luciferase reporter constructs from the 1.1kb *ITGB6* promoter reporter used by others [18] (See Figure 1A). With progressively decreasing size, transcription factor binding sites are lost from the reporter constructs. This series of reporter constructs was transfected in to iHBECs and luciferase activity measured after four hours. Loss of the most distal regions of the *ITGB6* promoter led to dramatic increases in basal promoter activity (Figure 1B). The increase in basal promoter activity was greatest when the -818 to -731 region of the promoter was absent suggesting this region contains important negative regulatory elements. *In silico* analysis using Transcription Element Search Software (University of Pennsylvania, USA) indicated binding sites for several transcription factors within the region -818 to -731. A schematic diagram showing this putative repressor region is shown in Figure 1C.

Elk1 Represses *ITGB6* and αvβ6 expression *in vitro*

Previous studies have suggested a role of ets family transcription factors in regulating the *ITGB6* promoter [18]. Therefore, we initially investigated the role of Elk1 in *ITGB6* regulation. Using chromatin immunoprecipitation (ChIP) we demonstrated that Elk1 bound to the putative repressor region of the *ITGB6* promoter in lung epithelial cells under basal conditions, but binding was not detected at a control region of the promoter upstream of the repressor region (Figure 2A). Site directed mutagenesis (SDM) of Elk1 binding motifs was utilised to investigate the effect of interrupting binding of Elk1 to the *ITGB6* promoter, which contains two Elk1 binding sites within the putative repressor region (at -772 and -752). ChIP was used to assess binding of endogenous Elk1 protein to the exogenous mutated and non-mutated promoter constructs. Binding of endogenous Elk1 to the exogenous, unmutated pGL3-*ITGB6* construct was detected, however, binding above an IgG control was not detected when either Elk1 site was mutated individually, or when both sites were mutated simultaneously (Figure 2B).

Following confirmation that Elk1 was unable to bind to the *ITGB6* promoter when the binding sites were mutated, the effect of mutating the Elk1 binding sites on promoter activity was assessed. Mutation of the Elk1 binding site at -772 resulted in a small but significant rise in *ITGB6* promoter activity, whereas mutation of the binding site at -752 had no significant effect (Figure 2C). However, mutation of both binding sites together resulted in a larger increase in *ITGB6* promoter activity, which was greater than the increase observed when the -772 site was mutated alone (Figure 2C).

To determine whether Elk1 repressed *ITGB6* mRNA expression we utilised an Elk1 siRNA that reduced Elk1 protein and mRNA expression compared with control siRNA (Figures 2D and 2E). Transfection of Elk1 siRNA increased basal *ITGB6* promoter activity (Figure 2F), suggesting a loss of basal transcriptional repression. Supporting these molecular studies

we demonstrated that Elk1 siRNA also led to a significant increase in αvβ6 cell surface protein levels two days following transfection (Figure 2G and H). These data demonstrate that Elk1 regulates *in vitro* lung epithelial cell αvβ6 integrin expression.

Glucocorticoid Receptor but not Progesterone Receptor, represses αvβ6 expression

The repressor region identified contains a number of putative transcription factor binding sites (Figure 1C), and the degree of repression mediated by Elk1 was considerably lower than observed by deletion of the complete repressor region. We therefore hypothesised that other transcription factors may be contributing to *ITGB6* repression. The receptors for glucocorticoids and progesterone had been identified in the repressor region therefore we investigated the effect of these hormones on *ITGB6* expression. Dexamethasone had no effect on basal activity of the full length *ITGB6* promoter after four hours but inhibited TGFβ1-induced increases in *ITGB6* promoter activity in iHBECs (Figure 3A). However, treatment of SAECs with dexamethasone for three days resulted in a reduction in cell surface expression of αvβ6 integrins (Figure 3B and 3C). Additionally a siRNA targeting GR, which reduced GR protein expression (Figure 3D), caused a small, but consistent increase in αvβ6 expression over two days (Figure 3E and 3F). Similar to dexamethasone, progesterone had no significant effect on basal *ITGB6* promoter activity but abrogated TGFβ1-induced increases in promoter activity (Figure 3G). However, progesterone had no significant effect on *ITGB6* mRNA expression over 24 hours (Figure 3H) nor had any effect on αvβ6 cell surface expression in SAECs over three days (Figure 3I). These data suggested that whilst glucocorticoids may affect *ITGB*6 expression, progesterone was unlikely to be involved in αvβ6 regulation.

Having identified two transcription factors (Elk1 and GR) capable of binding the putative repressor region, and repressing *ITGB6* and αvβ6 we sought to determine whether they co-regulated αvβ6 expression. Firstly, we utilised a combination of dexamethasone treatment, to activate GR, and Elk1 siRNA and assessed the effect on αvβ6 expression. Confirming the data shown in Figure 2G transfection of iHBECs with Elk1 siRNA caused a modest, but significant increase in αvβ6 expression (Figure 4A). However, activation of the GR with dexamethasone for two days did not effect αvβ6 levels in the presence of either control or Elk1 siRNA (Figure 4A). We next investigated the effect of dexamethasone treatment on TGFβ-induced increases in αvβ6 expression. Despite showing a trend towards reducing TGFβ-induced αvβ6 expression, dexamethasone did not have a statistically signficiant effect on TGFβ-induced increases in αvβ6 expression in iHBECs after two days (Figure 4B). Finally, iHBECs were transfected with Elk1 or GR siRNAs, or both siRNAs in combination and αvβ6 expression was measured in unstimulated and TGFβ stimulated cells. Both Elk1 and GR siRNA increased basal αvβ6 expression as previously shown, although transfection of both siRNAs did not act synergystically to further enhance αvβ6 cell surface levels (Figure 4C). Transfection with Elk1 and GR siRNA had a similar effect on TGFβ stimulated cells although the increase was not statistically significant compared with TGFβ alone (Figure 4C).

Loss of *Elk1 in vivo* results in an exaggerated response to bleomycin-induced lung fibrosis.

Although the *in vitro* data suggested that Elk1 was unlikely to mediate complete repression of the *ITGB6* promoter, we hypothesised that even moderate effects on epithelial αvβ6 integrin expression were likely to be important in response to injury. Therefore, to determine whether global loss of Elk1 was sufficient to increase *Itgb6* expression *in vivo* and exacerbate fibrotic responses we employed the bleomycin model of lung fibrosis. $Elk1^{-/0}$ male mice, and wild-type littermate controls ($Elk1^{+/0}$), were instilled with saline or 60 IU of bleomycin. Lungs were harvested 28 days following bleomycin instillation then lung fibrosis and *Itgb6* mRNA levels measured. Bleomycin increased levels of *Itgb6* mRNA in homogenates of $Elk1^{-/0}$ and $Elk1^{+/0}$ whole lung tissues, however, there were significantly greater levels of *Itgb6* mRNA in the lungs of $Elk1^{-/0}$ animals treated with bleomycin (Figure 5A).

Lung fibrosis was quantified by measuring levels of hydroxyproline in lung tissue and by assessing histology using Masson's trichrome stain. Bleomycin treatment increased lung hydroxyproline levels in both $Elk1^{+/0}$ and $Elk1^{-/0}$ animals, however, lungs from bleomycin treated

Elk1$^{-/0}$ mice contained significantly higher levels of hydroxyproline compared with bleomycin-treated *Elk1*$^{+/0}$ animals (Figure 5B). To confirm that deposition of collagen in the lungs, in response to bleomycin, was greater in *Elk1*$^{-/0}$ animals compared with control animals histological sections of lung tissue were stained with Masson's trichrome. Low power, stiched images of entire lung lobes demonstrate the patchy nature of lung fibrosis following bleomycin-induced lung injury but show that the areas of fibrosis were considerably larger in the *Elk1*$^{-/0}$ animals compared with the control animals (Figures 5C and D), and this was confirmed in high power images (Figure 5E i-iv). Lung tissue from these animals was also stained by immunohistochemistry for αvβ6 integrins. There was no evidence of αvβ6 protein expression in lungs of injured mice (data not shown) consistent with previous reports [25]. Although expression of the αvβ6 integrin was evident in areas of lung damage in both *Elk1*$^{-/0}$ and *Elk1*$^{+/0}$ animals, expression was markedly increased in the *Elk1*$^{-/0}$, compared with wild-type control, animals (Figure 5E, panels v and vii). Furthermore, *Elk1*$^{-/0}$ animals demonstrated a trend towards increased pSmad2 staining compared with *Elk1*$^{+/0}$ animals (Figure 5E, panels vi and viii and Figure 5F), consistent with reduced Elk1 expression leading to enhanced expression of TGFβ-activating integrins. Taken together these data support the hypothesis that Elk1 is capable of suppressing *Itgb6* mRNA and αvβ6 expression *in vivo*, and ultimately limit the fibrotic response to injury *in vivo*.

Having identified that GR could also repress *ITGB6 in vitro* we evaluated its effect *in vivo.* Animals were treated with bleomycin to induce lung injury and *Itgb6* expression, or saline, and received daily i.p. injections of either dexamethasone, or PBS control. *Itgb6* mRNA levels were determined in the lungs after 14 days. There was a clear trend towards reduced *Itgb6* expression in both the saline and bleomycin groups treated with daily dexamethasone compared with the groups treated with daily PBS (Figure 5G), however this did not reach statistical significance. However, when the data were analysed to determine any effect of dexamethasone irrespective of the presence of lung injury, there was a signficiant reduction in *Itgb6* mRNA following dexamethasone treatment compared with PBS treatment (p=0.01). This effect was also observed after 21 days of treatment (Data not shown). These data confirm the *in vitro* observations and show that Elk1 and GR can regulate expression of *Itgb6* in murine lung.

The repressor of *ITGB6* transcription, Elk1, is reduced in pulmonary fibrosis

Having identified that Elk1 is important for repression of *ITGB6* expression in the lung we hypothesised that this pathway would be dysregulated in human disease. We have previously demonstrated that *ITGB6* mRNA levels are increased in lung tissue homogenates from lung fibrosis patients [25], which we again found in this study (Figure 6A). ChIP analysis demonstrated that there was binding of Elk1 to the *ITGB6* promoter in non-fibrotic human lung tissue, and that there was reduced binding in samples from patients with PF (p=0.05; Figure 6B). It was clear that in some patients with pulmonary fibrosis (PF) there was no detectable binding of Elk1 to the *ITGB6* promoter above IgG controls (Figure 6B), therefore levels of Elk1 protein were assessed by western blot in fibrotic (PF) and non-fibrotic (NF) lung tissue samples. Elk1 was expressed in all control samples tested (Figure 6C and 6D) but levels were lower, and in some cases undetectable, in the PF tissue tested. Quantification of the western blot signals by densitometry demonstrated that PF patients had significantly less Elk1 protein than controls (Figure 6C). Specificity of the antibody used was confirmed by probing protein samples from both wild-type and Elk1$^{-/0}$ animals. Elk1 protein was detected in wild-type animals but not Elk$^{-/0}$ animals (Figure 6E).

To assess expression of Elk1 protein macroscopically in human lung tissue serial sections of paraffin wax embedded lung tissue from NF (n=4) and PF (n=4) donors were immunostained for both αvβ6 and Elk1. Confirming previous reports [13] NF donors had low levels of αvβ6 expression within the alveolar epithelium (Figure 6F, panel i). In contrast lung tissue from PF donors demonstrated marked expression of αvβ6 integrins within the alveolar epithelium that was particularly evident around regions of fibrosis (Figure 6F, panel ii). Supporting a role for Elk1 being responsible for negative regulation of αvβ6 integrins and confirming that Elk1 protein expression is aberrant in fibrosis we found that

NF donors had high levels of epithelial Elk1 protein expression whereas PF donors had minimal expression of Elk1 within the lung epithelium (Figure 6F, panels iii and iv).

DISCUSSION

IPF is a progressive lung disease with extremely poor survival and no effective treatments. Activation of TGFβ via αvβ6 integrins, the expression of which is limited to epithelial cells, is a fundamental process in the pathogenesis of pulmonary fibrosis [7, 9, 11]. Interactions between lung epithelial cells and fibroblasts, partly through epithelial cell αvβ6-mediated TGFβ activation, are now thought to be critical to the development and progression of lung fibrosis [4]. A common observation in IPF patients, and in other fibrotic disorders, is the upregulation of αvβ6 integrins within the epithelium [9, 12, 14, 15]. The molecular mechanisms driving enhanced αvβ6 expression, however, have not been fully investigated. In the current study we have highlighted a region of the *ITGB6* gene promoter responsible for repressing basal gene transcription and, fundamentally, have identified a novel and important function for the transcription factor Elk1 in fibrosis as a negative regulator of *ITGB6* and αvβ6 integrins in epithelial cells.

Previous studies have suggested that recombinant Elk1 reduces activity of an exogenous *ITGB6* promoter in the HEK293 cell line [18]. Our data are consistent with these findings and demonstrates that loss of Elk1, or interruption of the ability of Elk1 to bind to the *ITGB6* promoter, increases *ITGB6* promoter activity in lung epithelial cells, which can, in turn, lead to increased expression of αvβ6 integrins *in vitro*. Furthermore, loss of Elk1 *in vivo* increased levels of *Itgb6* mRNA and collagen deposition in a bleomycin model of lung fibrosis. Elk1 is a ternary complex factor that is commonly thought of as an activator of transcription when phosphorylated. However, it also contains an R domain that can potently repress transcription [27]. The role of Elk1 phosphorylation has not yet been determined on *ITGB6* expression, and may prove to be an important regulatory pathway. It is possible that dynamic regulation between unphosphorylated Elk, which functions as a transcriptional repressor, and phosphorylated Elk1, which can act as an transcriptional activator may be important in repair processes and is the focus of current studies.

Other putative repressors of the *ITGB6* promoter highlighted by *in silico* analysis included the progesterone and glucocorticoid receptors (PR and GR). Both PR and GR are expressed in the normal lung [28, 29], and although the effects of progesterone on *ITGB6* expression have not previously been investigated either in the lung or in other tissues, it has been shown to modulate the expression of several other integrin subunits including the α2 and α11 subunits, as well as α1β1 and αvβ3 integrins in the placenta and endometrium [30, 31]. However, we found no convincing effect of progesterone on basal *ITGB6* promoter activity, *ITGB6* mRNA levels or cell surface αvβ6 integrin levels suggesting that PR is not a major contributor to repression of *ITGB6* in lung epithelial cells.

In contrast with progesterone, dexamethasone was able to inhibit TGFβ1-induced *ITGB6* promoter activity, inhibit basal αvβ6 integrin cell surface expression, and showed a trend towards reducing TGFβ-induced αvβ6 expression *in vitro*. Furthermore, confirming our *in vitro* data, activation of GR *in vivo* using daily treatment with dexamethasone led to reduced expression of *Itgb6* mRNA in the lung. Although it is possible that the inhibition of αvβ6 integrin expression is a direct result of GR activation with consequent binding to, and repression of, the *ITGB6* promoter we do not favour this hypothesis. Our data suggest that dexamethasone does not affect basal promoter activity, but rather inhibits TGF-β1-induced *ITGB6* expression. Furthermore, it has previously been shown that GR can interrupt TGFβ/Smad3 mediated plasminogen activator inhibitor-1 (PAI1) expression via a physical interaction with the transcriptional activation functions of Smad3 [32]. Since TGF-β has been postulated as a positive regulator of αvβ6 integrins via an autocrine loop [17] we would hyothesise that GR reduces αvβ6 protein expression via interrupting this TGF-β-mediated autocrine loop.

The effect of siRNA-induced knock-down of Elk1 on both *ITGB6* promoter activity and αvβ6 integrin expression was relatively modest and the effect of Elk1 siRNA does not completely recapitulate the effect of loss of the entire repressor region from the *ITGB6* promoter.

Efficiency of the siRNA transfection is unlikely to have reached 100%, which is supported by the observation that transfection with Elk1 siRNA did not result in 100% knock-down of Elk1 protein or mRNA. Furthermore, the process of transfecting cells *in vitro* can often result in cell injury [33, 34] and it is well documented that epithelial cell injury results in upregulation of αvβ6 [8, 12], which raises the possibility that transfection of the control siRNA might have increased αvβ6 expression therefore reducing the effect "window" observed when comparing Elk1 siRNA with control siRNA. Unfortunately, the nuclear location of Elk1 made alternative methods of inhibiting Elk1 difficult.

Importantly, the repressor region of the *ITGB6* promoter contains multiple transcription factor binding sites, many of which may also exhibit repressor functions on the *ITGB6* gene. In addition to Elk1 our data highlight a potential role for GR in suppressing αvβ6 expression *in vitro* and *Itgb6 in vivo*. However, inhibiting the actions of Elk1 and GR simultaneously did not have co-regulatory effects on αvβ6 expression over and above inhibiting either transcription factor singularly. It is, therefore, very likely that other factors that may bind the repressor region are involved in the basal repression of the *ITGB6* gene, some of which may have combined additive effects on αvβ6 repression. However, it is beyond the scope of this manuscript to investigate them all. Fundamentally, a consistent effect of manipulating Elk1 on *ITGB6* promoter, gene and protein expression was observed both *in vitro* and *in vivo* highlighting a novel and important function for Elk1 in *ITGB6* regulation.

These data are the first description of aberrant Elk1 expression in any fibrotic condition, and could help explain the mechanisms driving enhanced αvβ6 expression in IPF. Elk1 is an X-linked gene (ensemble.org), thus defects in a single allele will have greater effects in males, which may potentially explain the gender differences associated with IPF [35]. Levels of Elk1 protein were reduced in lung tissue homogenates and histological sections from PF patients, and furthermore, tissue ChIP analysis revealed that Elk1 binding to the *ITGB6* promoter was reduced in PF tissue. Expression of *ITGB6* is restricted to epithelium, therefore these data suggest that a defect in total lung Elk1 is likely to play a role in de-repression of epithelial αvβ6 integrin expression observed in fibrotic lung tissue. While the mechanisms regulating decreased Elk1 expression in pulmonary fibrosis are unclear, Elk1 is a target of mir-185 (microRNA.org), which is increased in lung tissue from patients suffering from rapidly progressive IPF [36].

While the focus of this manuscript is on the effect of Elk1 on expression of epithelial restricted αvβ6 integrins it is apparent that global loss of Elk1 expression in IPF could have implications on multiple pathways in many other cells types involved in the pathogenesis of IPF. Elk1 signalling has been implicated in regulating elastin expression in lung fibroblasts [37], hyaluronan expression in dermal fibroblasts [38], and alpha-smooth muscle actin in vascular smooth muscle cells [39], all of which could have implications for fibrogenesis. Our own *in silico* analysis has highlighted the presence of Elk1 binding motifs in the promoters of several fibrotic genes including alpha smooth muscle actin, the β8 integrin subunit and TGFβRII amongst others. Loss of Elk1 may also impact expression of other integrins, such as the αvβ1 integrin, which has recently been implicated in fibrogenesis in multiple organs [40].

We propose a novel function for the transcription factor Elk1 in fibrosis. Following epithelial cell injury Elk1 serves to limit the repair response through repression of *ITGB6* transcription and therefore αvβ6 integrins. Where there is failure of repression through, in part, loss/interruption of Elk1 expression/signalling, fibrogenesis may ensue through enhanced and sustained expression of *ITGB6.* In summary, this study describes a novel function for the transcription factor Elk1 in contributing to repression of αvβ6 integrins in the pulmonary epithelium and show that reduced Elk1-mediated repression is important in driving the increased αvβ6 integrin expression and collagen deposition observed in pulmonary fibrosis.

ACKNOWLEDGEMENTS

We would like to thank Professor Derek Mann and Dr Jelena Mann (Newcastle University, UK) for providing advice about performing ChIP on tissue, and to Dr Karl Deacon (University of Nottingham, UK) for invaluable expert advice about performing SDM and assessing binding of Elk1 to exogenous plasmid constructs by ChIP.

We would also like to thank research staff and nurses at all research centres for their help with collection and processing of human tissue.

This work was supported by the following research awards: Wellcome Trust (grant 085350 to G.J.), the National Centre for the Replacement, Refinement and Reduction of Animals in Research (grant G110564 to G.J.), the German Cancer Aid (grant 109886 to A.N.), and an NC3Rs David Sainsbury Fellowship held by A.T.

CONFLICT OF INTERESTS

Dr Gisli Jenkins has received consultancy fees from Glaxosmithkline, Intermune, Boehringer Ingelheim, Biogen and PharmAkea, and lecture fees from Intermune, Medimmune, Roche and Boehringer Ingelheim. Dr Jenkins also has sponsored research agreements with Glaxosmithkline, Novartis and Biogen. Drs. Shelia Violette and Paul Weinreb are share holders of Biogen Idec.

AUTHOR CONTRIBUTIONS

ALT conceived and coordinated the study, performed all experiments except those listed below, and wrote the manuscript. AH assisted in conducting all of the *in vivo* animal studies, and performed the histochemical staining of mouse lung tissue. JP performed the immunohistochemical staining of human lung tissue shown in Fig. 5, performed quantification of the pSmad2 immunohistochemistry and assisted with αvβ6 flow cytometry. AEJ performed the *in* vivo dexamethasone study and provided microscopy expertise for obtaining data in Figs. 4 and 5. AS assisted in conducting the *in vivo* animal studies. EH conducted the SDM to produce *ITGB6* promoter constructs with mutated Elk1 binding sites. CK-L performed the progesterone promoter studies in Fig. 2. SMV and PHW created and provided the αvβ6 blocking antibody, and contributed to manuscript preparation. AJK, GL, HP and PJW were involved in experimental design and critique of the manuscript. WW contributed to experimental design, manuscript critique, and provided the human lung tissue from IPF patients. SA and AH were involved in the studies using Elk1 null animals (Fig. 4). GJ conceived the study and co-wrote the manuscript. All authors have approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1: The ITGB6 Gene Promoter Contains a Large Repressor Region

A: Schematic diagram demonstrating the series of deletion mutants of the full length pGL3-ITGB6 promoter luciferase reporter construct

B: iHBECs were transfected with the either empty pGL3 vector, full length *ITGB6* promoter reporter or a series of *ITGB6* promoter deletion mutants. After four hours luciferase activity was measured to determine promoter activity. Data expressed as mean relative firefly/renilla ratio ± SEM from three independent experiments. * = $p < 0.05$

C: *In silico* analysis revealed the presence of several transcription factor binding sites between -818 and -731 from the TSS. This schematic diagram shows these transcription factor binding sites.

Figure 2: αvβ6 is repressed by Elk1 binding to the *ITGB6* promoter at –772 and –752

A: Basal binding of Elk1 to the repressor region of the endogenous *ITGB6* promoter (-934 to -753) and a control region (-1604 to -1497) was assessed by ChIP in iHBECs. Binding of Elk1 above IgG binding levels was detected at the repressor region but not at the control region. Data are expressed as mean relative binding (relative to IgG) ± SEM from three independent experiments. * = $p < 0.05$

B: SDM of Elk1 sites located at either -772, or -752 or both was performed. H647 cells were transfected with either non-mutated pGL3-*ITGB6* reporter or either of the mutated constructs for 24 hours. ChIP was performed for endogenous Elk1. Relative binding above IgG was calculated relative to the full length, non-mutated promoter construct. Figure shows mean relative binding ± SEM from three independent experiments. ** = $p < 0.005$

C: SDM of Elk1 sites located at either -772, or -752 or both was performed. iHBECs were transfected with these constructs and luciferase activity measured. Data expressed as mean relative firefly/renilla ratio ± SEM from three indepdent experiments. * = $p < 0.05$

D: iHBECs were transfected with Elk1 or control siRNA and Elk1 expression measured by western blotting. Figure is representative of three independent experiments. The ratio of Elk1: GAPDH as calculated by densitometry is shown beneath the bands.

E: iHBECs were transfected with Elk1 or control siRNA and *ELK1* mRNA assessed. Data expressed as mean *ELK1* expression ± SEM from two independent experiments. * = $p < 0.05$

F: iHBECs were cotransfected with Elk1 or control siRNA with the full length *ITGB6* promoter reporter. Data expressed as mean relative firefly/renilla ± SEM from three independent experiments. * = $p < 0.05$

G: iHBECs were transfected with either control siRNA or Elk1 siRNA and αvβ6 expression assessed after two days. Grey line = negative control; black solid line = control siRNA; dotted line = Elk1 siRNA. Histogram is representative of three independent experiments

H: Bar chart shows amalgamated data from experiments shown in Figure 2G expressed as fold change MFI. * = $p < 0.05$

Figure 3: Glucocorticoid Receptor, but not Progesterone Receptor, represses αvβ6

A: iHBECs transfected with the *ITGB6* promoter reporter (black bars) or an empty vector control (white bars) were stimulated with 2 ng/ml TGFβ1, 10 μM dexamethasone or TGFβ1 and dexamethasone together. Data expressed as mean relative firefly/ renilla ratio ± SEM from three independent experiments. * = $p < 0.05$

B: SAECs were stimulated with dexamethasone for three days and αvβ6 assessed. Grey line = negative control. Solid line = 0 μM. Dotted line = 10 μM. Histogram is representative of three independent experiments.

C: The data shown in Figure 3B are expressed as a bar chart. Figure shows amalgamated data from three independent experiments expressed as fold change MFI. ** = $p < 0.01$

D: iHBECs were transfected with control or GR siRNA and GR protein expression determined by western blot to confirm GR knockdown. Figure is representative of three independent experiments. . The ratio of GR: GAPDH as calculated by densitometry is shown beneath the bands.

E: iHBECs were transfected with control or GR siRNA and αvβ6 assessed after two days. Grey line = negative control; black solid line = control siRNA; black dotted line = GR siRNA. Histogram is representative of three independent experiments.

F: The data shown in Figure 3E are expressed as a bar chart. Figure shows amalgamated data from three independent experiments expressed as fold change MFI. ** = $p < 0.01$

G: iHBECs were transfected with either an *ITGB6* promoter reporter (black bars) or an empty vector control (white bars) and stimulated with TGFβ1 (2 ng/ml), progesterone (10 μM) or TGFβ1 and progesterone in combination. Data are expressed as mean relative firefly/renilla ratio ± SEM from three independent experiments. ** = $p < 0.01$

H: iHBECs were stimulated with 10μM progesterone and *ITGB6* mRNA measured by QPCR over 24 hours. Data are expressed as mean fold change relative to 0 hour ± SEM from three independent experiments.

I: SAECs were stimulated with 10 μM progesterone and αvβ6 was assessed. Grey line = negative control. Solid line = 0 μM. Dotted line = 10 μM progesterone. Histogram is representative of three independent experiments.

Figure 4: Inhibiting the Actions of Elk1 and GR Simulataneously Does Not have an Additive Effect on αvβ6 Repression

A: iHBECs were transfected with Elk1 siRNA for 24 hours then stimulated with either 0 or 10μM dexamethasone. αvβ6 expression was determined after two days. Figure shows amalgamated data from three independent experiments. Data are expressed as fold change in MFI. * = $p<0.05$

B: iHBECs were left unstimulated or stimulated with 2ng/ml TGFβ, 10μM dexamethasone or both agonists in combination for three days. αvβ6 expression was determined by flow cytometry. Figure shows amalgamated data from three independent experiments. Data are expressed as fold change in MFI. * = $p<0.05$

C: iHBECs were transfected with either control siRNA, Elk1 siRNA, GR siRNA or both siRNAs in combination for 24 hours. The cells were then treated with 0 or 2ng/ml TGFβ for two days. αvβ6 expression was measured by flow cytometry. Figure shows amalgamated data from three independent experiments. Data are expressed as fold change in MFI. * = $p<0.05$, ** = $p<0.01$

Figure 5: Loss of *Elk1 in vivo* results in an exaggerated response to bleomycin-induced lung fibrosis.

A: *Itgb6* mRNA levels were measured in lung homogenates by QPCR and were significantly elevated in bleomycin instilled $Elk1^{-/0}$ mice (n = 5 mice), compared with bleomycin-treated $Elk1^{+/0}$ control mice (n = 7 mice). * = $p < 0.05$

B: Total lung collagen, assessed by hydroxyproline levels, was significantly elevated in bleomycin instilled $Elk1^{-/0}$ mice (n = 5 mice) compared with $Elk1^{+/0}$ controls (n = 7 mice). * = $p < 0.05$

C: Lung collagen was stained using Masson's trichrome. Nikon NIS Elements was utilised to image an entire lung lobe from an $Elk^{+/0}$ animal to demonstrate the deposition of collagen across the lobe. Figure shows a representative single lung lobe from an $Elk^{+/0}$ animal treated with bleomycin (n = 4 mice).

D: Lung collagen was stained using Masson's trichrome. Nikon NIS Elements was utilised to image an entire lung lobe from an $Elk1^{-/0}$ animal to demonstrate the deposition of collagen across the lobe. Figure shows a representative single lung lobe from an $Elk1^{-/0}$ animal treated with bleomycin (n = 4 mice).

E: Histological sections of lung from $Elk1^{-/0}$ and $Elk1^{+/0}$ animals following a bleomycin model of lung fibrosis were stained with Masson's trichrome and for αvβ6 and pSmad2 by immunohistochemistry. All images were acquired using Nikon NIS Elements and are representative of a minimum of 3 (maximum of 5) animals. i) Saline treated $Elk1^{+/0}$ animal stained with Masson's trichrome. ii) Saline treated $Elk1^{-/0}$ animal stained with Masson's trichrome. iii) Bleomycin treated $Elk1^{+/0}$ animal stained with Masson's trichrome. iv) Bleomycin treated $Elk1^{-/0}$ animal stained with Masson's trichrome. v) Bleomycin treated $Elk1^{+/0}$ animal immunostained for αvβ6. vi) Bleomycin treated $Elk1^{+/0}$ animal immunostained for pSmad2. vii) Bleomycin treated $Elk1^{-/0}$ animal immunostained for αvβ6. viii) Bleomycin treated $Elk1^{-/0}$ animal immunostained for pSmad2.

F: Histological sections of lung from $Elk1^{-/0}$ and $Elk1^{+/0}$ animals following a bleomycin model of lung fibrosis were stained immunohistochemically for pSmad2 (see Figure 5E vi and viii). pSmad2

positive nuclei were quantified. Data are expressed as mean brown nuclei per field of view in bleomycin-treated $Elk1^{+/0}$ (n=4) and $Elk1^{-/0}$ (n=5) animals.

G: Animals were instilled with either bleomycin or saline and treated with either PBS or dexamethasone daily. *Itgb6* mRNA levels were measured in lung homogenates by QPCR after 14 days and were significantly elevated in bleomycin instilled PBS treated mice (n = 12 mice), compared with saline instilled PBS treated mice (n = 7 mice). * = $p < 0.05$

Figure 6: Expression of Elk1 is reduced in pulmonary fibrosis

A: Relative expression of *ITGB6* mRNA in NF (n = 6 donors) and PF (n = 6 donors) human lung tissue was assessed by QPCR. Data are expressed as relative mRNA expression compared with NF samples. Line demonstrates median.

B: Binding of Elk1 to the *ITGB6* promoter in NF and PF tissue measured by ChIP. Data expressed as relative binding to the *ITGB6* promoter compared with IgG control and the median expressed. Relative binding of 1 or below demonstrates no binding to the promoter above IgG.

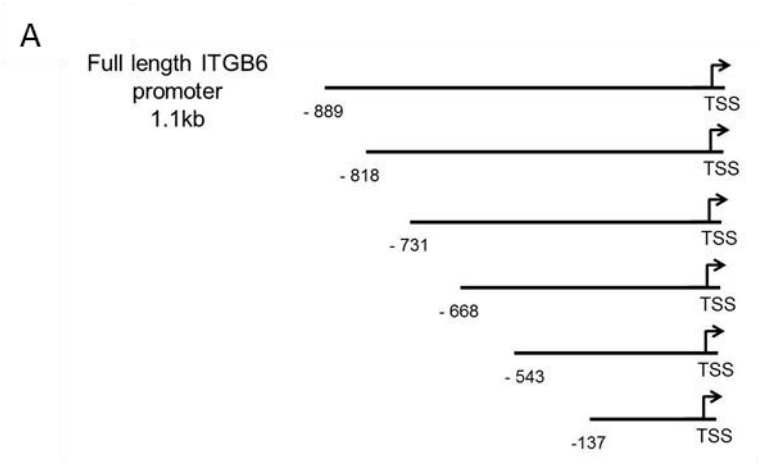
C: Densitometry analysis of Elk1 expression was in whole lung extracts from NF (n = 8 donors) and PF (n = 14 donors) donors. Data expressed as a ratio of Elk1 band intensity: GAPDH band intensity. ** = $p < 0.01$

D: Representative immunoblot showing Elk1 and GAPDH expression in NF (n = 4 donors) and PF (n = 5 donors) lung tissue. The ratio of Elk1: GAPDH as calculated by densitometry is shown beneath the bands.

E: Immunoblot showing Elk1 and GAPDH protein expression in $Elk1^{+/0}$ (n=2 mice) and $Elk1^{-/0}$ (n=2 mice) animals. The ratio of Elk1: GAPDH as calculated by densitometry is shown beneath the bands.

F: Histological sections of lung from control (n = 4 donors) and PF (n = 4 donors) donors was immunostained for αvβ6 and Elk1. All images were acquired using Nikon NIS Elements and are representative of the donor samples used. i) Lung tissue from a control donor was stained for αvβ6. ii) Lung tissue from a PF donor was stained for αvβ6. iii) Lung tissue from a control donor was stained for Elk1. iv) Lung tissue from a PF donor was stained for Elk1.

Figure 1


A
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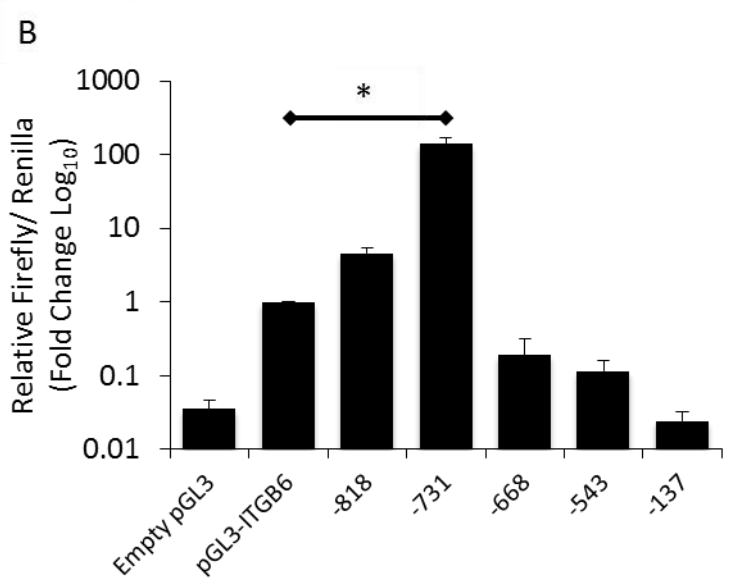
TSS
- 818
TSS
- 731
TSS
- 668
TSS
- 543
TSS
-137
TSS


B
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pGL3-ITGB6
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-731
-668
-543
-137

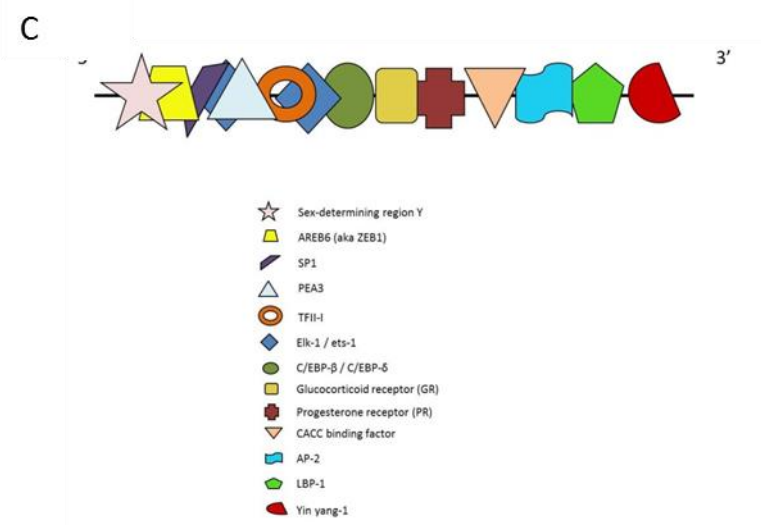

C
3'
Sex-determining region Y
AREB6 (aka ZEB1)
SP1
PEA3
TFII-I
Elk-1 / ets-1
C/EBP-β / C/EBP-δ
Glucocorticoid receptor (GR)
Progesterone receptor (PR)
CACC binding factor
AP-2
LBP-1
Yin yang-1

Figure 2

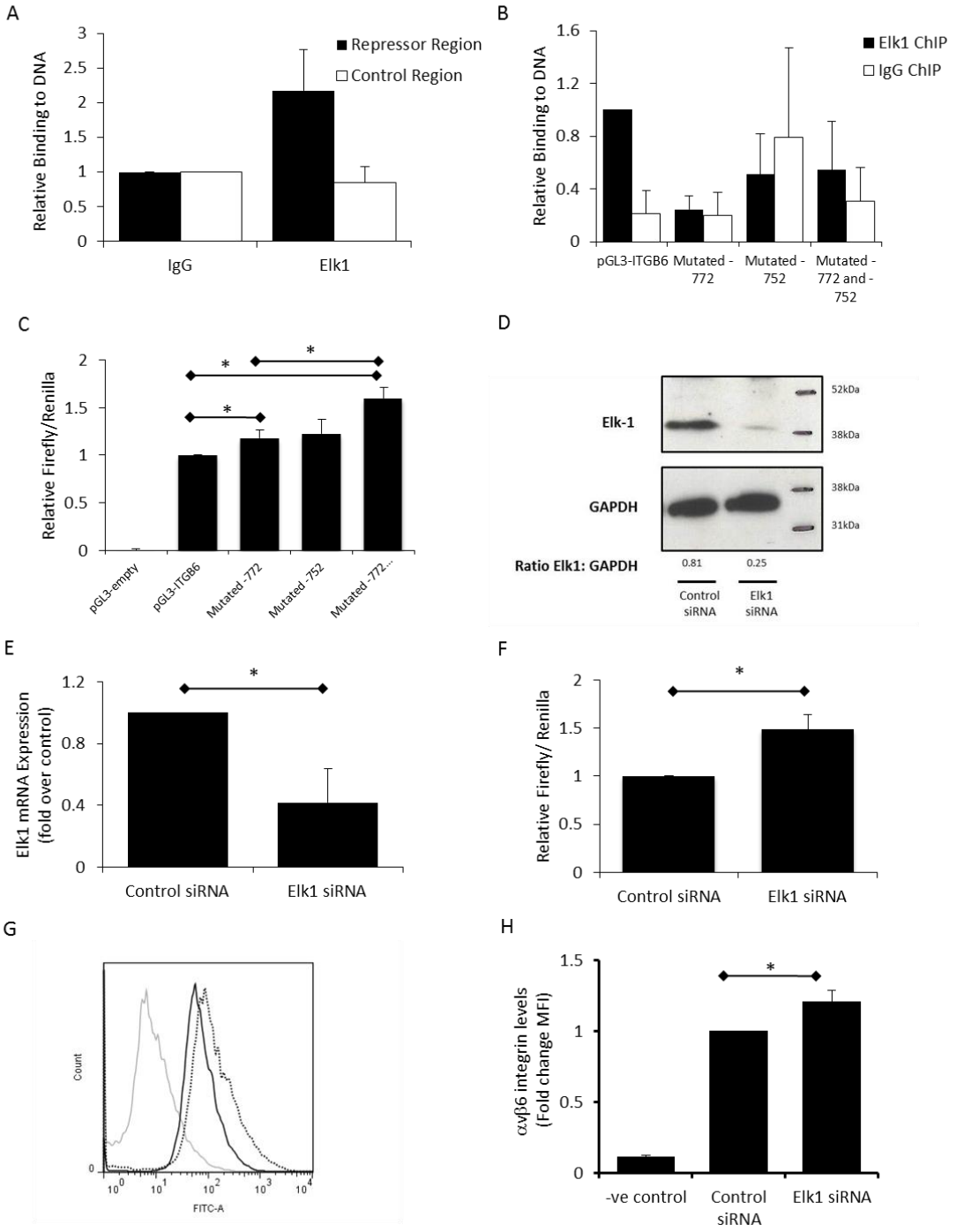

A
Relative Binding to DNA
Repressor Region
Control Region
IgG
Elk1
B
Relative Binding to DNA
Elk1 ChIP
IgG ChIP
pGL3-ITGB6
Mutated -772
Mutated -752
Mutated -772 and -752
C
Relative Firefly/Renilla
pGL3-empty
pGL3-ITGB6
Mutated -772
Mutated -752
Mutated -772...
D
Elk-1
GAPDH
52kDa
38kDa
38kDa
31kDa
Ratio Elk1: GAPDH
0.81
0.25
Control siRNA
Elk1 siRNA
E
Elk1 mRNA Expression (fold over control)
Control siRNA
Elk1 siRNA
F
Relative Firefly/ Renilla
Control siRNA
Elk1 siRNA
G
Count
FITC-A
H
αvβ6 integrin levels (Fold change MFI)
-ve control
Control siRNA
Elk1 siRNA

Figure 3

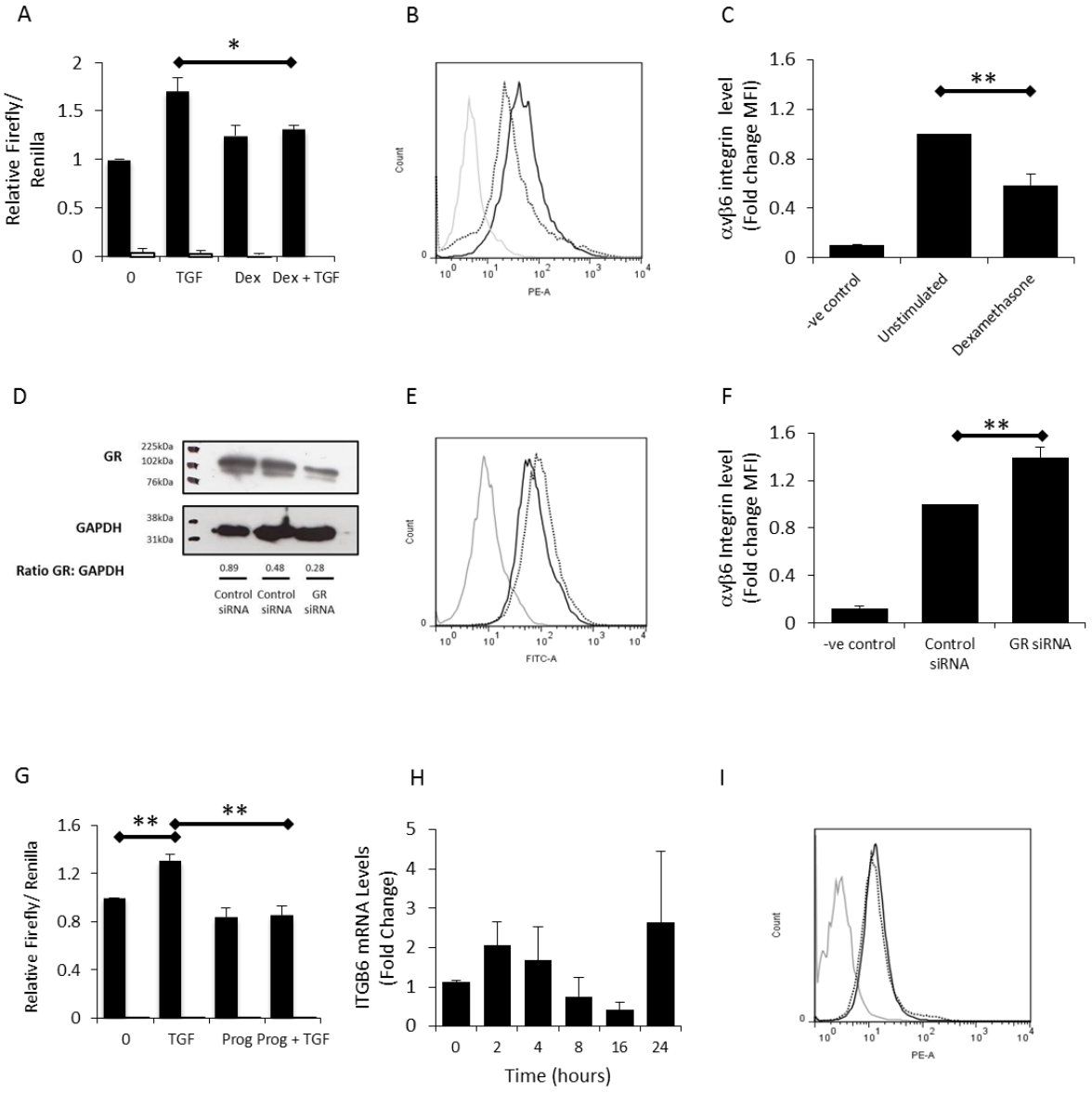

A
Relative Firefly/ Renilla
2
1.5
1
0.5
0
*
0
TGF
Dex
Dex + TGF
B
Count
0
PE-A
C
αvβ6 integrin level (Fold change MFI)
1.6
1.2
0.8
0.4
0
**
-ve control
Unstimulated
Dexamethasone
D
GR
225kDa
102kDa
76kDa
GAPDH
38kDa
31kDa
Ratio GR: GAPDH
0.89
0.48
0.28
Control siRNA
Control siRNA
GR siRNA
E
Count
0
FITC-A
F
αvβ6 Integrin level (Fold change MFI)
1.6
1.2
0.8
0.4
0
**
-ve control
Control siRNA
GR siRNA
G
Relative Firefly/ Renilla
1.6
1.2
0.8
0.4
0
**
**
0
TGF
Prog
Prog + TGF
H
ITGB6 mRNA Levels (Fold Change)
5
4
3
2
1
0
0
2
4
8
16
24
Time (hours)
I
Count
0
PE-A

Figure 4

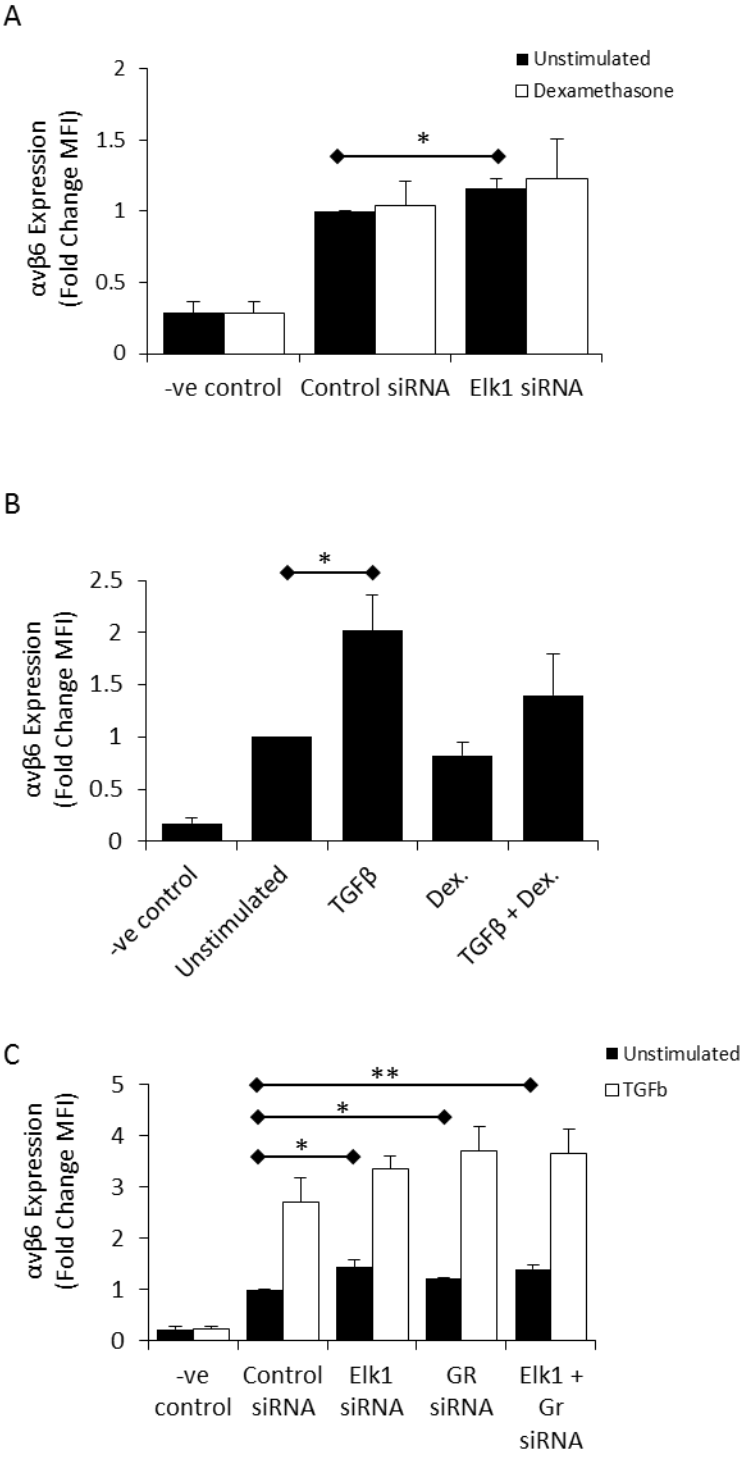

A
Unstimulated
Dexamethasone
αvβ6 Expression (Fold Change MFI)
2
1.5
1
0.5
0
*
-ve control
Control siRNA
Elk1 siRNA
B
αvβ6 Expression (Fold Change MFI)
2.5
2
1.5
1
0.5
0
*
-ve control
Unstimulated
TGFβ
Dex.
TGFβ + Dex.
C
Unstimulated
TGFb
αvβ6 Expression (Fold Change MFI)
5
4
3
2
1
0
**
*
*
-ve control
Control siRNA
Elk1 siRNA
GR siRNA
Elk1 + Gr siRNA

Figure 5

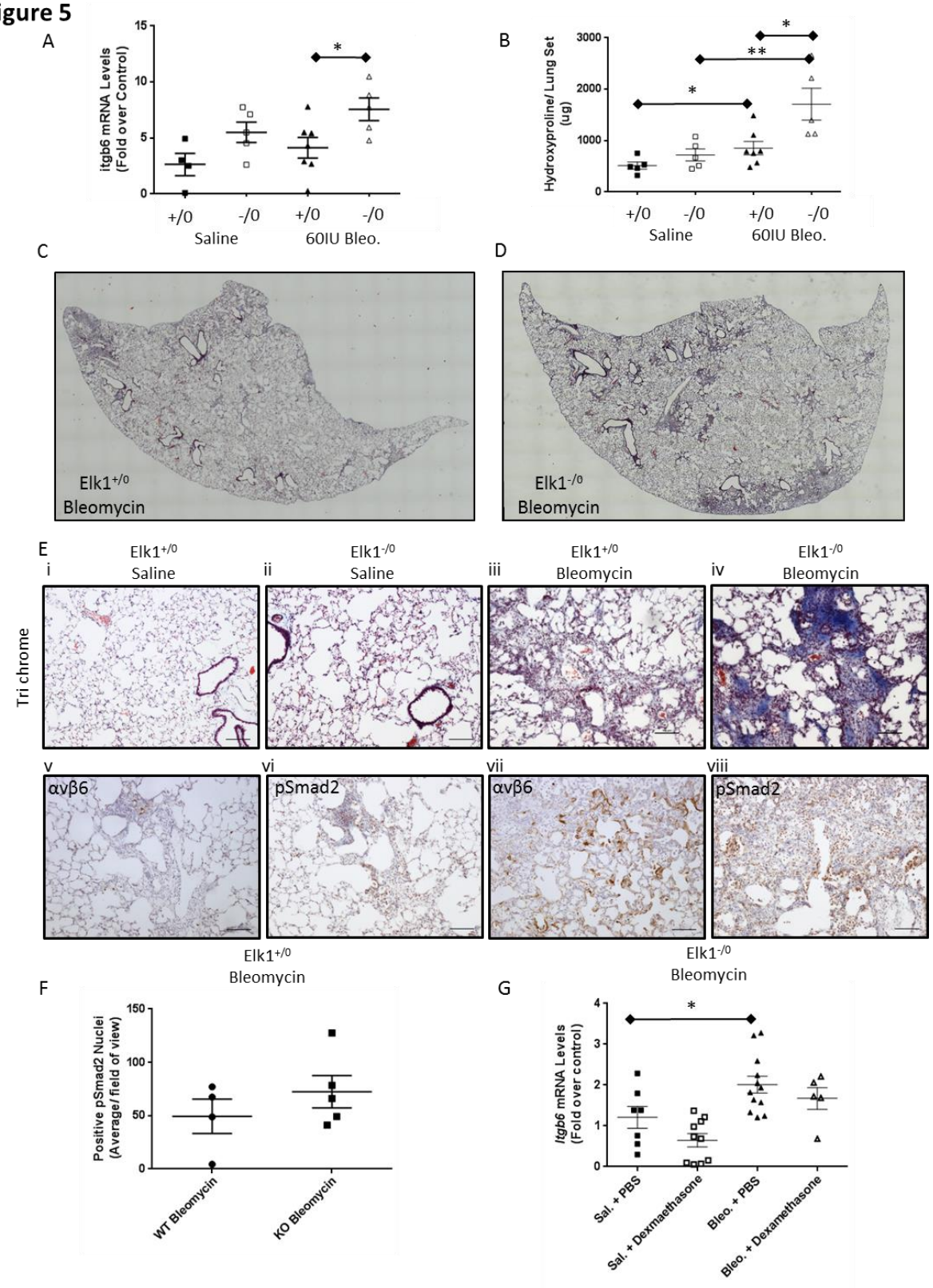
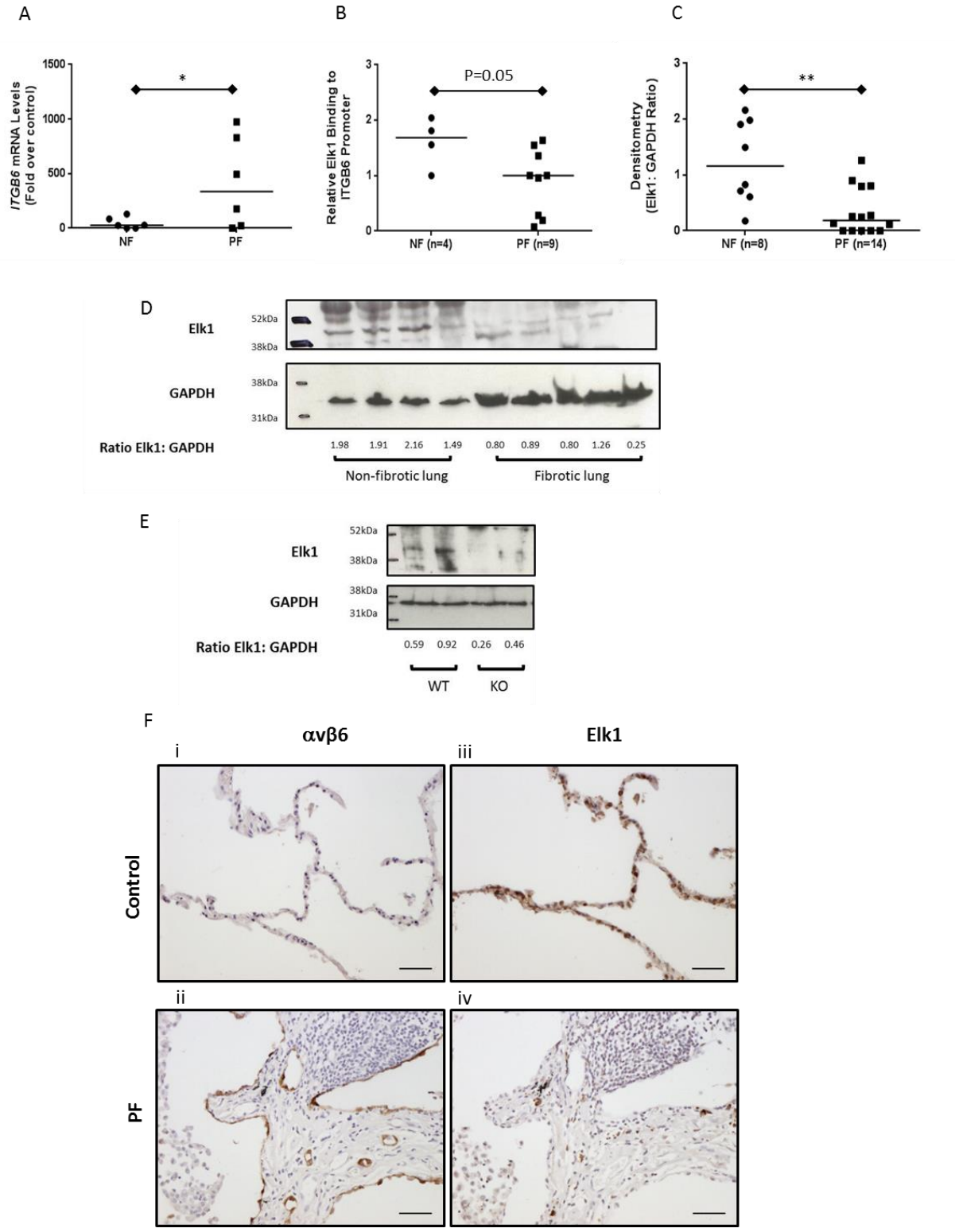

A
itgb6 mRNA Levels (Fold over Control)
+/0
-/0
+/0
-/0
Saline
60IU Bleo.
B
Hydroxyproline/ Lung Set (ug)
C
Elk1+/0
Bleomycin
D
Elk1-/0
Bleomycin
E
Tri chrome
i Elk1+/0 Saline
ii Elk1-/0 Saline
iii Elk1+/0 Bleomycin
iv Elk1-/0 Bleomycin
v αvβ6
vi pSmad2
vii αvβ6
viii pSmad2
F
Elk1+/0 Bleomycin
Positive pSmad2 Nuclei (Average/ field of view)
WT Bleomycin
KO Bleomycin
G
Elk1-/0 Bleomycin
Itgb6 mRNA Levels (Fold over control)
Sal. + PBS
Sal. + Dexmaethasone
Bleo. + PBS
Bleo. + Dexamethasone

Figure 6


A
B
C
ITGB6 mRNA Levels (Fold over control)
1500
1000
500
0
*
NF
PF
Relative Elk1 Binding to ITGB6 Promoter
3
2
1
0
P=0.05
NF (n=4)
PF (n=9)
Densitometry (Elk1: GAPDH Ratio)
3
2
1
0
**
NF (n=8)
PF (n=14)
D
Elk1
52kDa
38kDa
GAPDH
38kDa
31kDa
Ratio Elk1: GAPDH
1.98 1.91 2.16 1.49 0.80 0.89 0.80 1.26 0.25
Non-fibrotic lung
Fibrotic lung
E
Elk1
52kDa
38kDa
GAPDH
38kDa
31kDa
Ratio Elk1: GAPDH
0.59 0.92 0.26 0.46
WT
KO
F
αvβ6
Elk1
i
iii
ii
iv
Control
PF